Molecular Cell
Article

Secreted Monocytic miR-150 Enhances Targeted Endothelial Cell Migration

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DOI 10.1016/j.molcel.2010.06.010

SUMMARY

MicroRNAs (miRNAs) are a class of noncoding RNAs that regulate target gene expression at the posttranscriptional level. Here, we report that secreted miRNAs can serve as signaling molecules mediating intercellular communication. In human blood cells and cultured THP-1 cells, miR-150 was selectively packaged into microvesicles (MVs) and actively secreted. THP-1-derived MVs can enter and deliver miR-150 into human HMEC-1 cells, and elevated exogenous miR-150 effectively reduced c-Myb expression and enhanced cell migration in HMEC-1 cells. In vivo studies confirmed that intravenous injection of THP-1 MVs significantly increased the level of miR-150 in mouse blood vessels. MVs isolated from the plasma of patients with atherosclerosis contained higher levels of miR-150, and they more effectively promoted HMEC-1 cell migration than MVs from healthy donors. These results demonstrate that cells can secrete miRNAs and deliver them into recipient cells where the exogenous miRNAs can regulate target gene expression and recipient cell function.

INTRODUCTION

MicroRNAs (miRNAs) are a class of noncoding RNAs consisting of processed products approximately 22 nucleotides in length that regulate gene expression in plants and animals (Ambros, 2004; Bartel, 2004). In a previous study, we showed that miRNAs are stably expressed in animal serum/plasma and that their unique expression patterns serve as "fingerprints" of various diseases (Chen et al., 2008). Mitchell et al. and two other groups reported that circulating miRNAs are promising biomarkers for prostate cancer, pregnancy, and ovarian cancer (Mitchell et al., 2008; Gilad et al., 2008; Resnick et al., 2009). By characterizing serum miRNA expression profiles under normal physiological conditions and in various disease states, we found that serum miRNAs are derived not only from circulating blood cells but also from other tissues directly affected by disease (Chen et al., 2008). Interestingly, unlike miRNAs extracted from tissues or cells, miRNAs extracted from sera are resistant to RNase A digestion (Chen et al., 2008), suggesting that serum miRNAs might be modified differently from tissue or cellular miRNAs. Although it has been speculated that serum miRNA may be released from broken cells (Mitchell et al., 2008), the mechanisms that regulate miRNA release and the potential biological functions of serum miRNAs are completely unknown.

Microvesicles (MVs) are small vesicles that are shed from almost all cell types under both normal and pathological conditions (Cocucci et al., 2008; Théry et al., 2002). These secreted MVs bear surface receptors/ligands of the original cells and have the potential to selectively interact with specific target cells (Théry et al., 2002). Cell-derived MVs may thus be ideal miRNA carriers and may also provide a mechanism for transport and exchange of miRNAs among nonadjacent cells. Recent studies identified miRNAs in different types of MVs derived from cultured cells (Skog et al., 2008; Valadi et al., 2007), supporting the idea that MVs, particularly exosomes, may serve as physiological carriers of miRNAs. Although Skog et al. (2008) described the expression profile of miRNAs in MVs derived from glioblastoma cells and Valadi et al. (2007) demonstrated the regulation of target genes in recipient cells by exosomes, there is no direct evidence to show that exogenous miRNAs delivered by MVs regulate the expression of target genes and cellular functions in recipient cells. Because MVs also contain proteins and other nucleotides (Théry et al., 2002; Skog et al., 2008; Valadi et al., 2007), their effects on recipient cells might be caused by molecules other than miRNAs.

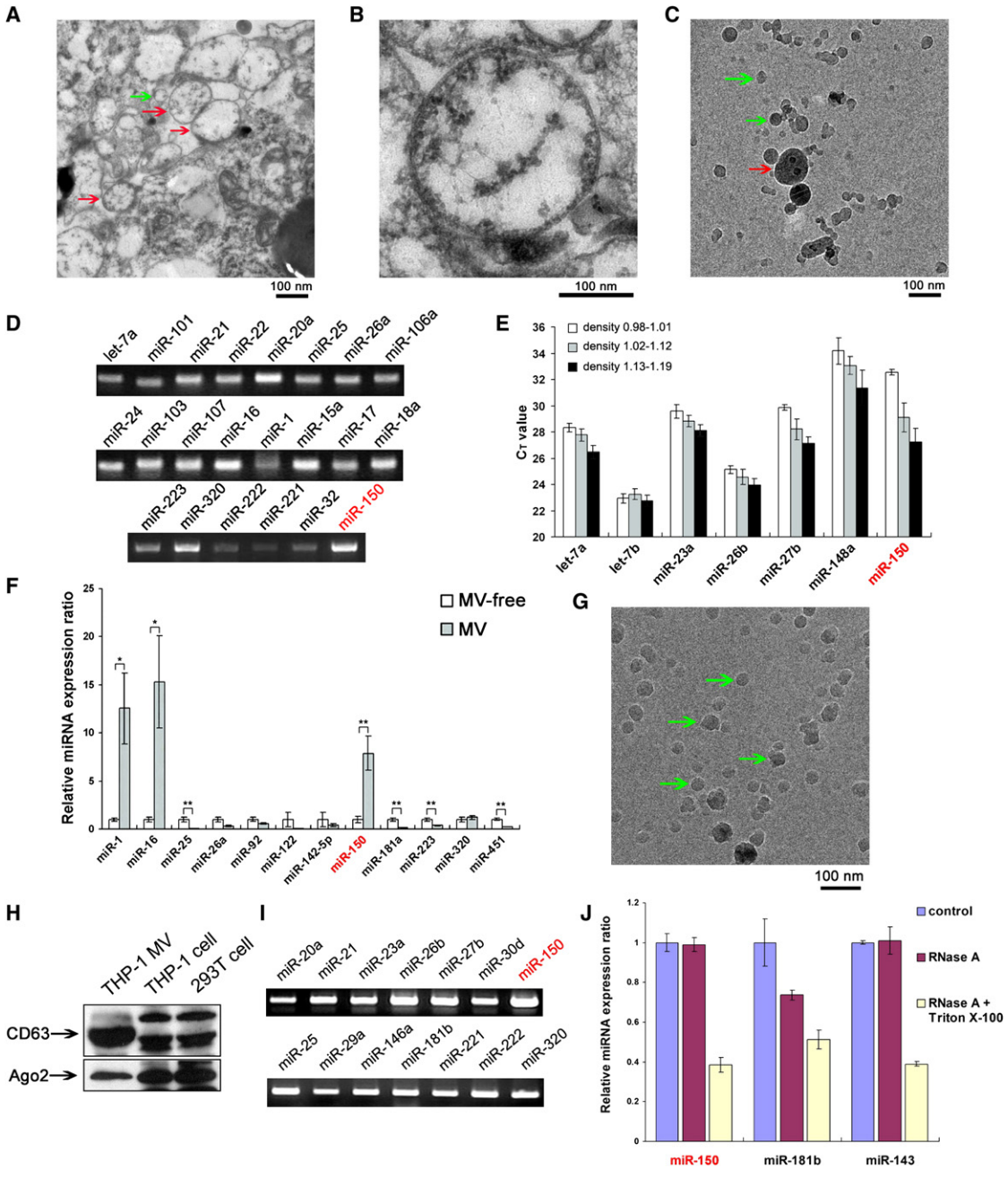


Figure 1. Characterization of miRNAs in MVs

(A and B) TEM micrographs of the MVs isolated from plasma of normal human subjects.

(C) Cryo-EM micrograph of the MVs isolated from plasma of normal human subjects. MVs consistent with the size of microparticles or exosomes were highlighted using red arrows or green arrows, respectively.

(D) Semiquantitative RT-PCR of miRNAs in MVs isolated from plasma of normal human subjects.

(E) Comparison of miRNA expression in MVs of different densities derived from OptiPrep-based density gradient centrifugation. miRNA expression levels were determined by quantitative RT-PCR, and the raw C_T values of each fraction were plotted. Results are presented as means ±SEM of three independent experiments.

(F) Comparison of miRNA expression in MV-free plasma and in the MV fraction. For comparison, the expression levels of miRNAs in MV-free plasma were arbitrarily set at 1. The y axes show arbitrary units representing relative miRNA expression levels. Results are presented as means ±SEM of three independent experiments (*$p < 0.05$, **$p < 0.01$).

(G) Cryo-EM micrographs of MVs isolated from culture medium of THP-1 cells. MVs consistent with the size of exosomes were highlighted using green arrows.

(H) Equal amounts of proteins from MVs and whole cells were analyzed by western blotting for exosome-enriched protein CD63 and a key protein involved in miRNA homeostasis, AGO2.

Furthermore, while there is no report of a possible carrier of serum miRNA, it is important to understand the potential mechanism of action of serum miRNA.

In this study, we further examine the sources of serum miRNAs and show that cells can selectively package miRNAs into MVs and actively secrete them into the circulation and into cell culture medium. We also demonstrate that these secreted miRNAs can be delivered into target cells and can modulate the biological functions of these cells via repression of target gene expression.

RESULTS

MVs Derived from Human Plasma and Cultured THP-1 Cells Contain miRNAs

In order to determine whether MVs are carriers of circulating miRNA, MVs were obtained from human plasma of healthy donors by sequential centrifugation (detailed experimental procedure is shown in a schematic diagram in Figure S5). Under electron microscopy, the isolated MVs appeared as cluster of vesicles of 40–200 nm in diameter, and each of those vesicles was surrounded by a double-layer membrane (Figures 1A–1C). They contained various miRNAs (Figure 1D). Using OptiPrep-based density gradient centrifugation, the MVs were separated into three fractions based on density in the range of 0.98–1.19 g/ml. All three fractions of MVs (0.98–1.01, 1.02–1.12, and 1.13–1.19 g/ml) contained miRNAs; the fraction with the highest density (1.13–1.19 g/ml) had the highest concentration of miRNAs (lower C_T value) (Figure 1E). The ratio of miRNA levels in MVs to that in MV-free plasma was compared. The majority of circulating miRNAs were present in MVs (Figure 1F). miR-150, miR-16, and miR-1 were mainly stored in MVs, whereas miR-25, miR-181a, miR-223, and miR-451 were present at relatively higher levels in MV-free plasma (Figure 1F). Furthermore, MVs isolated from culture medium of THP-1 cells were characterized. They were identified by their morphologically uniform vesicular structure in electron microscopy (around 30–60 nm) (Figure 1G) and by exosomal marker protein CD63 (Figure 1H). MVs derived from cultured THP-1 cells also contained various miRNAs, including monocytic miR-150 (Figure 1I). Interestingly, such vesicles also contain some proteins required for miRNA activity, such as Argonaute 2 (AGO2) (Gibbings et al., 2009), albeit less than that in whole-cell lysates (Figure 1H). These results show that MVs derived from both plasma and cultured cells contain miRNAs and suggest that MVs may be the major carriers of circulating miRNAs.

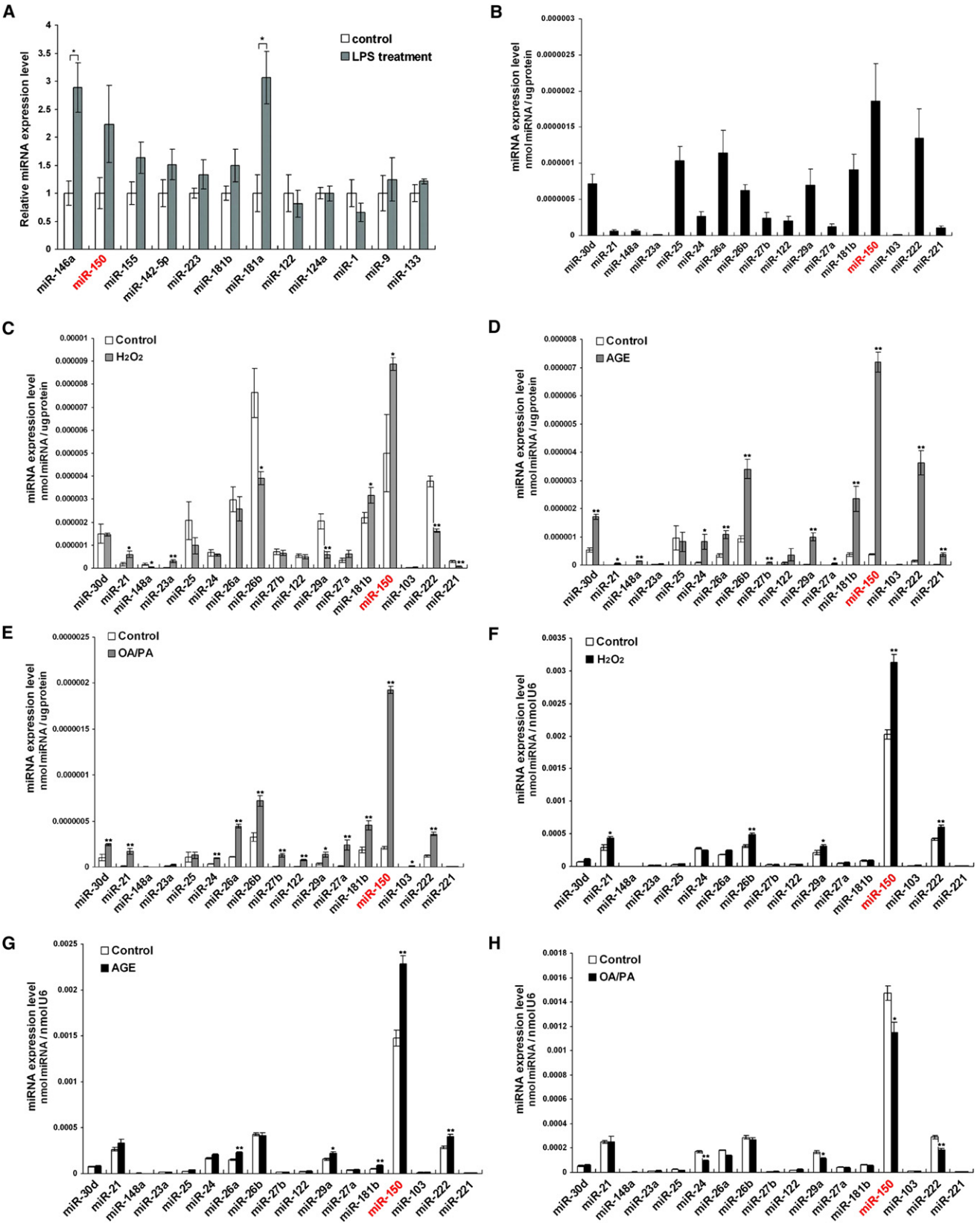
To further characterize the miRNAs in MVs, we established a method to calculate the absolute expression levels of miRNAs in MVs. Using diluted synthetic miRNA as template, we first showed that our quantitative RT-PCR assay was able to monitor miRNA levels specifically and sensitively (Figure S1A). To validate that our method of normalizing miRNA expression to total protein content among MVs is reasonable, a series of MVs (1/1, 1/2, 1/4, 1/8, 1/16, or 1/32) were subjected to quantitative RT-PCR to analyze miRNA levels. Simultaneously, BCA method was employed to quantify total protein content in MVs. Decreasing the MV input led to a corresponding decrease in the levels of both miRNAs (Figure S1B) and proteins (Figure S1C), with a Pearson correlation coefficient (R) between miRNA and protein levels close to 1 (Figures S1D and S1E). The results suggest that our method is an accurate and reproducible tool to analyze miRNA expression in MVs. By this method, we demonstrated that the miRNAs were indeed present within the MVs but not simply being released as a contaminant during the MV purification process, as the miRNA expression levels in MVs treated with RNase were little changed compared to those in untreated MVs, while miRNA levels in MVs treated with both RNase and Triton X-100 significantly reduced (Figure 1J).

Blood Cells and Cultured THP-1 Cells Selectively Package miRNAs into MVs in Response to Various Stimuli

In order to assess the specificity of cellular secretion of miRNAs, miRNAs present in MVs derived from LPS-treated human blood cells were analyzed. When normalized to total protein content, miR-150 and other immune-related miRNAs, including miR-146a and miR-181a, were markedly upregulated in MVs derived from LPS-treated blood compared to those from untreated blood (Figure 2A). In contrast, other miRNAs, such as miR-122 and miR-124a, were unchanged in MVs in response to LPS stimulation (Figure 2A). Cultured THP-1 cells were used for further study. The absolute expression levels of miRNAs in untreated THP-1 MVs were determined (Figure 2B). The most highly enriched miRNA in THP-1 MVs was miR-150, which was present at a concentration of approximately 0.002 pmol/μg total protein). The levels of various miRNAs in MVs were generally 0.02%–0.5% of their cellular contents (Table S1). THP-1 cells were then stimulated with LPS, oleic acid (OA)/palmitic acid (PA), advanced glycation end products (AGEs), and H_2O_2. LPS not only stimulated the release of MVs from THP-1 cells (Figure S2), which is consistent with a previous report (Aras et al., 2004), but also altered the types and levels of miRNAs in THP-1 MVs (data not shown). Furthermore, although OA/PA, AGEs, and H_2O_2 affected the expression pattern and levels of miRNAs both in cells (Figures 2F–2H) and in MVs (Figures 2C–2E), MVs showed remarkably greater changes in their specific miRNA content than overall cells did. While certain stimulations increased miR-30d, miR-26a, miR-26b, miR-29a, miR-181b, and miR-222 levels in THP-1 MVs, all three stimuli caused a significant elevation of miR-150 levels in secreted MVs (Figures 2C–2E). A summary of the alteration of miRNAs in MVs and cells in response to three stimuli is listed in Table S2. These results clearly demonstrate that blood cells and cultured THP-1 cells actively and selectively package miRNAs into MVs and secrete them into the circulation or the culture medium in response to various stimuli.

(I) Semiquantitative RT-PCR of miRNAs in MVs isolated from culture medium of THP-1 cells.

(J) Quantitative RT-PCR analysis of miRNAs in MVs treated with RNase or Triton X-100. MVs were isolated from culture medium of THP-1 cells and divided into three equal portions. These samples were then untreated (control) or treated with RNase or RNase plus 0.1% Triton X-100 for 30 min. Quantitative RT-PCR assay was performed to determine the retained miRNAs in each sample. Results are presented as means ±SEM of three independent experiments (see also Figure S1).


A
Relative miRNA expression level
control
LPS treatment
miR-146a
miR-150
miR-155
miR-142-5p
miR-223
miR-181b
miR-181a
miR-122
miR-124a
miR-1
miR-9
miR-133
B
miRNA expression level
nmol miRNA / ugprotein
C
Control
H2O2
D
Control
AGE
E
Control
OA/PA
F
Control
H2O2
miRNA expression level
nmol miRNA / nmol U6
G
Control
AGE
H
Control
OA/PA
miR-30d
miR-21
miR-148a
miR-23a
miR-25
miR-24
miR-26a
miR-26b
miR-27b
miR-122
miR-29a
miR-27a
miR-181b
miR-150
miR-103
miR-222
miR-221

THP-1 MVs Deliver miRNAs into Recipient HMEC-1 Cells

To determine the biological functions of secreted miRNAs, cultured human microvascular endothelial HMEC-1 cells were used as the recipients of MVs derived from THP-1 cells. DiI-C16-labeled MVs isolated from THP-1 cell culture medium rapidly entered into the cultured HMEC-1 cells at 37°C (Figure 3A). However, the internalization of MVs into HMEC-1 cells was blocked by incubation at 4°C (Figure 3A). A similar process of internalization of MVs derived from 293T cells into HMEC-1 cells was observed (Figures S3A and S3B). Interestingly, labeling of HMEC-1 cells by fluorescently labeled THP-1 MVs was dose dependent (Figures S3C and S3D). Furthermore, if THP-1 cells were labeled with DiI-C16 for only 10 min, the isolated MVs did not label targeted HMEC-1 cells (Figure S3E). This result was not unexpected, since only a very limited amount of labeled MVs was secreted in such a short time frame. These results demonstrate that labeling HMEC-1 cells is not due to DiI-C16 carryover but to the internalization of MVs into targeted cells, which is an active process depending on temperature, incubation time, and the amount of MVs. Together, our results show that every cell type tested can act as a recipient of MVs secreted by human monocyte/macrophage cells.

Subsequently, we directly fluorescently tagged a synthetic miRNA oligonucleotide (FITC-tagged miR-150) and transfected it into THP-1 cells. Figures S3F–S3H showed that the FITC-tagged miR-150 was efficiently transfected into THP-1 cells. We then isolated MVs from THP-1 cell culture medium, added the MVs to HMEC-1 cells, and looked for the fluorescently labeled oligonucleotide in the HMEC-1 cells. Figure 3B showed that HMEC-1 cells treated with MVs bearing FITC-tagged miR-150 were fluorescently labeled under fluorescence microscopy (indicated by arrows). By flow cytometry, uptake and retention of the FITC-tagged miR-150 in HMEC-1 cells was demonstrated (Figure 3C). These results clearly demonstrate that miRNAs can be secreted and delivered into target cells via MVs.

miR-150, a leukocyte- and lymphocyte-specific miRNA, was abundantly expressed in THP-1 cells (Figure 3D, left panel) and THP-1-derived MVs (Figure 2B). In contrast, HMEC-1 cells and 293T cells had significantly lower levels of miR-150 (Figure 3D, left panel). Figure 3E also showed that miR-150 was enriched in THP-1 cells and THP-1 MVs. THP-1 MVs increased miR-150 levels in HMEC-1 cells nearly 12-fold, while MVs derived from control 293T cells had no effect on the level of miR-150 expression in HMEC-1 cells (Figure 3D, right panel). On the other hand, 293T MVs contained high levels of miR-24* (Figure 3D, left panel), and miR-24* was elevated in HMEC-1 cells when HMEC-1 cells were treated with 293T MVs (Figure 3D, right panel). THP-1-derived MVs, which contained low levels of miR-24*, had no effect on its expression in HMEC-1 cells (Figure 3D, right panel). Moreover, increasing the incubation time for uptake of THP-1 MVs led to a corresponding increase in miR-150 levels in HMEC-1 cells (Figure S3I). The results strongly suggest that the uptake of miR-150 into HMEC-1 cells is a kinetics process. The pre-miR-150 levels in HMEC-1 cells were unchanged by treatment with either THP-1 or 293T MVs (Figure 3F), providing further evidence that the increased miR-150 levels in HMEC-1 cells were due to direct delivery by THP-1 MVs rather than to induction of miR-150 by cell-MV interactions. Taken together, these results show that miR-150 was directly delivered to HMEC-1 cells by THP-1 MVs.

Exogenous miR-150 Reduces c-Myb Protein Level in HMEC-1 Cells

The protein level of c-Myb, a gene targeted by miR-150 (Lin et al., 2008; Xiao et al., 2007), was determined in order to assess the biological function of exogenous miR-150 delivered by THP-1 MVs in HMEC-1 cells. As shown in Figure 3G, the expression level of c-Myb in HMEC-1 cells was significantly reduced following incubation with THP-1 MVs, while MVs from 293T cells had no effect on c-Myb protein expression. In order to determine the specificity of this effect and to exclude the possibility that the decreased c-Myb protein was caused by factors other than miR-150 in THP-1 MVs, the following experiments were performed. First, miR-150 expression was knocked down in THP-1 cells using antisense oligonucleotides. Efficient interference of miR-150 was shown in Figures S3K and S3L. MVs from miR-150-deficient THP-1 cells had no effect on c-Myb protein expression (Figure 3H). Second, HMEC-1 cells were directly treated with pre-miR-150, anti-miR-150, or other unrelated miRNAs, followed by assessment of c-Myb levels. Efficient overexpression of miR-150 following transfection with pre-miR-150 is shown in Figures S3J and S3L. Overexpression of miR-150 in HMEC-1 cells significantly decreased c-Myb expression. By contrast, treatment with anti-miR-150 had almost no effect on c-Myb expression (Figure 3H). This result was not unexpected, since the endogenous level of miR-150 in HMEC-1 cells is quite low. No apparent effect of miR-16 overexpression on c-Myb level was seen (Figure 3H). Third, 293T cells were transfected with pre-miR-150, and MVs from miR-150-sufficient 293T cells were harvested. Treatment with these MVs significantly decreased c-Myb

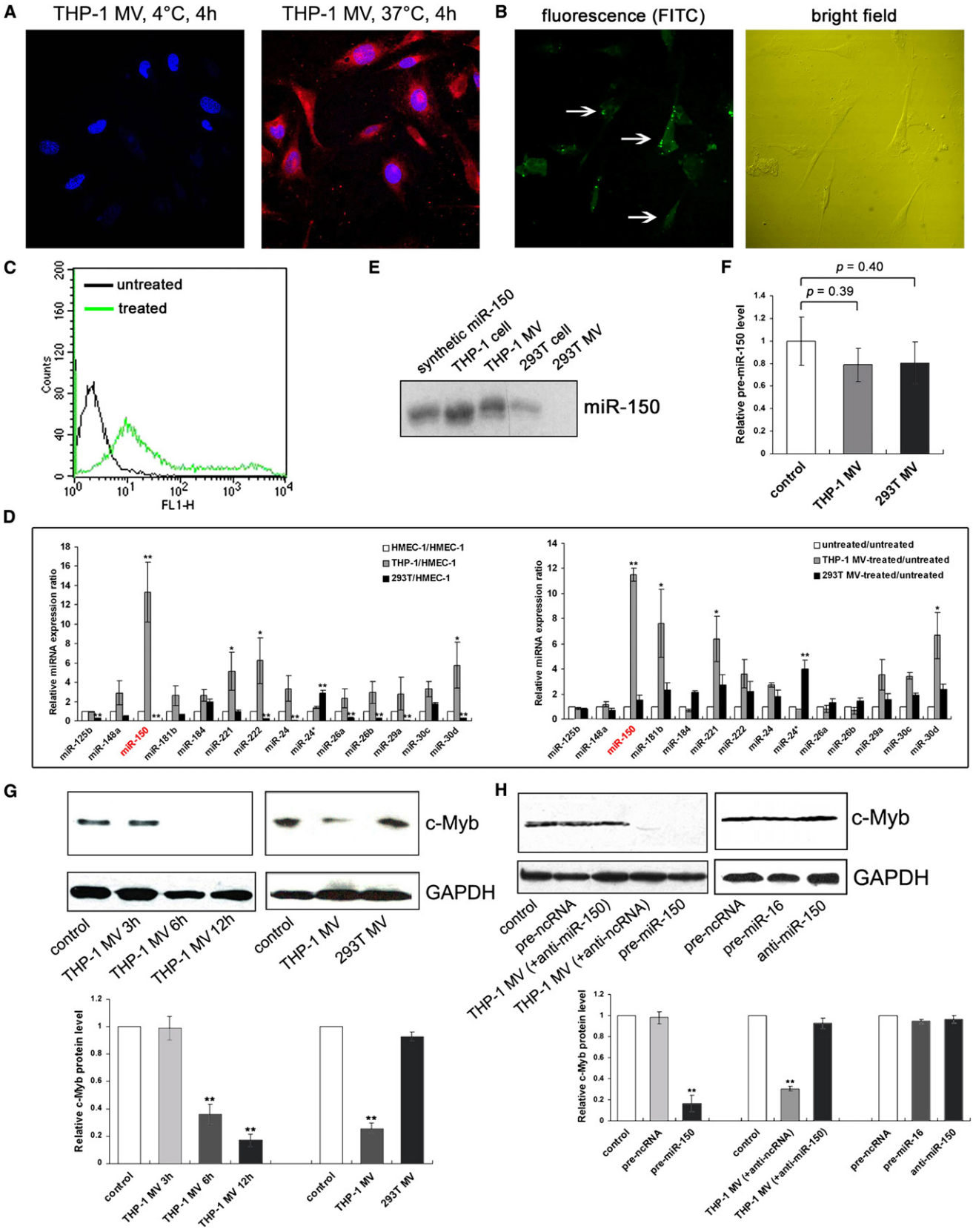
Figure 2. Selective Packaging of miRNAs into MVs under Stimulation Conditions

(A) Comparison of miRNA expression in MVs isolated from normal human blood treated with or without 100 ng/ml LPS (37°C, 1 hr). miRNA expression levels in MVs were determined by quantitative RT-PCR. Results are presented as means ±SEM of three independent experiments (*$p < 0.05$, LPS-treated MVs versus untreated control MVs).

(B) Quantitative RT-PCR analysis of the absolute expression levels of miRNAs in THP-1 MVs. Results are presented as means ±SEM of three independent experiments.

(C–E) Effects of H_2O_2, AGE, and OA/PA on miRNA expression in THP-1 MVs. THP-1 cells were stimulated with or without 50 ng/ml H_2O_2 (C), 1 mg/ml AGE (D), or 400 μmol/l OA/PA (E). MVs were isolated from culture medium, and miRNA expression levels in THP-1 MVs were determined by quantitative RT-PCR. Results are presented as means ±SEM of five independent experiments (*$p < 0.05$, **$p < 0.01$, treated samples versus untreated samples).

(F–H) Effects of H_2O_2, AGE, and OA/PA on miRNA expression in THP-1 cells. THP-1 cells were stimulated by H_2O_2 (F), AGE (G), or OA/PA (H) as described for (C)–(E), and cellular miRNA expression levels were determined by quantitative RT-PCR. Results are presented as means ±SEM of five independent experiments (*$p < 0.05$, **$p < 0.01$, treated samples versus untreated samples) (see also Figure S2 and Tables S1 and S2).


A
THP-1 MV, 4°C, 4h
THP-1 MV, 37°C, 4h
B
fluorescence (FITC)
bright field
C
untreated
treated
Counts
FL1-H
E
synthetic miR-150
THP-1 cell
THP-1 MV
293T cell
293T MV
miR-150
F
p = 0.40
p = 0.39
Relative pre-miR-150 level
control
THP-1 MV
293T MV
D
HMEC-1/HMEC-1
THP-1/HMEC-1
293T/HMEC-1
Relative miRNA expression ratio
untreated/untreated
THP-1 MV-treated/untreated
293T MV-treated/untreated
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miR-148a
miR-150
miR-181b
miR-184
miR-221
miR-222
miR-24
miR-24*
miR-26a
miR-26b
miR-29a
miR-30c
miR-30d
G
c-Myb
GAPDH
control
THP-1 MV 3h
THP-1 MV 6h
THP-1 MV 12h
THP-1 MV
293T MV
Relative c-Myb protein level
H
pre-ncRNA
THP-1 MV (+anti-miR-150)
THP-1 MV (+anti-ncRNA)
pre-miR-150
pre-miR-16
anti-miR-150

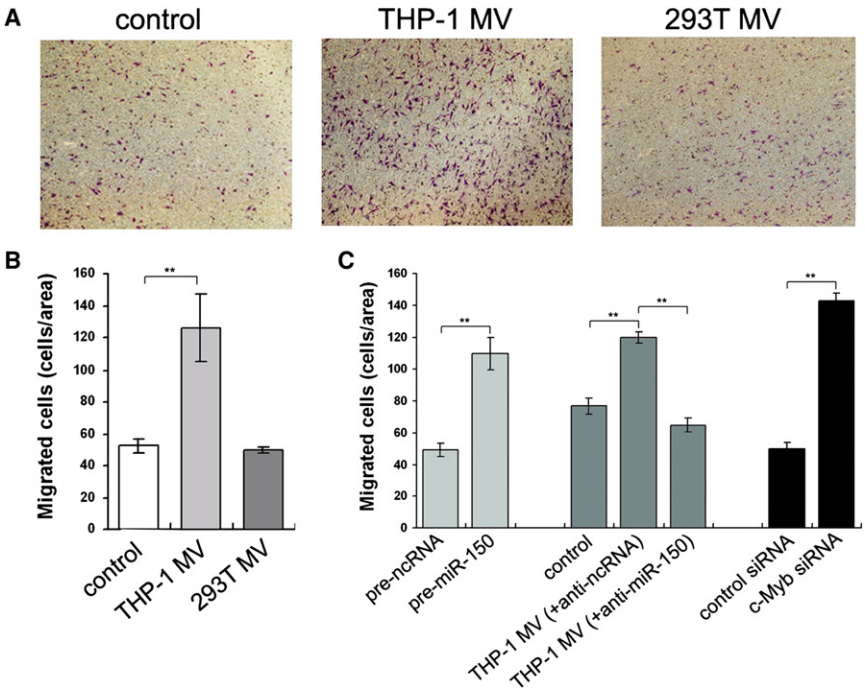

Figure 4. Exogenous miR-150 Enhances Recipient HMEC-1 Cell Migration

(A) Photoimages of Transwell analysis of HMEC-1 cells that were treated with/without THP-1 or 293T MVs. Results are representative data from three independent experiments.

(B) Migration rates of HMEC-1 cells that were treated with/without THP-1 or 293T MVs. Results are presented as means ±SEM of three independent experiments (**$p < 0.01$, treated HMEC-1 versus untreated HMEC-1).

(C) Migration rates of HMEC-1 cells that were untreated (lane 3) or treated with pre-miR-150 (lane 2), scrambled negative control RNAs (lane 1), miR-150-deficient THP-1 MVs (lane 5), normal THP-1 MVs (lane 4), c-Myb siRNA (lane 7), or control of siRNAs (lane 6). Results are presented as means ±SEM of five independent experiments (**$p < 0.01$) (see also Figure S4).

expression in HMEC-1 cells (Figure S3M). Finally, to demonstrate the direct repression of c-Myb by THP-1 MVs through elements in the 3′ UTR, the entire c-Myb 3′ UTR was cloned into a luciferase reporter plasmid, and the resulting plasmid was transfected into HMEC-1 cells combined with or without treatment of THP-1 MVs. As shown in Figure S3N, the luciferase reporter activity was significantly reduced following incubation with THP-1 MVs. In contrast, MVs from miR-150-deficient THP-1 cells had no effect on luciferase reporter activity. Taken together, the results suggest that miR-150 secreted by THP-1 cells can effectively get into HMEC-1 cells and reduce c-Myb expression in HMEC-1 cells.

Exogenous miR-150 Enhances Recipient HMEC-1 Cell Migration

As a transcription factor, *c-Myb* is critical to various cellular functions, including cell lineage commitment, proliferation, differentiation, and migration (Xiao et al., 2007; Lin et al., 2008; Zhao et al., 2009; Kopecki et al., 2007). The migration capacity of HMEC-1 cells following incubation with THP-1 MVs was assessed. THP-1 MVs strongly enhanced HMEC-1 cell migration across Transwell filters (Figure 4A). The migration rate of HMEC-1 cells treated with THP-1 MVs was about 2.5-fold higher than that of untreated cells or of cells treated with 293T MVs (Figure 4B). Moreover, consistent with the increased expression of miR-150 in THP-1 MVs upon exposure to AGE (Figure 2D), the migration rate of HMEC-1 cells treated with MVs isolated from AGE-stimulated THP-1 cells was higher than that treated with unstimulated MVs (Figure S4A). Furthermore, miR-150-deficient THP-1 MVs had no effect on migration

Figure 3. Exogenous miR-150 Reduces c-Myb Protein Level in HMEC-1 Cells

(A) Confocal microscopy image of the internalization of fluorescently labeled MVs into HMEC-1 cells. THP-1 and 293T cells were labeled with DiI-C16 (red) and then cultured in RPMI 1640 medium supplemented with 10% FBS. After 4 hr, the supernatants were collected and centrifuged to harvest MVs. The THP-1 or 293T MVs were resuspended in MCDB-131 medium and incubated with HMEC-1 cells at 4°C or 37°C, respectively. After incubation for 2 hr, HMEC-1 cells were washed, fixed, and observed under confocal microscopy.

(B) Images of HMEC-1 cells treated with MVs bearing FITC-tagged miR-150. HMEC-1 cells were incubated with MVs bearing FITC-tagged miR-150 for 6 hr and viewed by fluorescent confocal microscopy.

(C) HMEC-1 cells were incubated with MVs bearing FITC-tagged miR-150 for 6 hr and then gated and analyzed on a flow cytometer. Untreated HMEC-1 cells served as a negative control.

(D) Quantitative RT-PCR analysis of miRNA expression in HMEC-1, THP-1, and 293T cells (left panel) and in HMEC-1 cells treated with or without THP-1 or 293T MVs for 12 hr (right panel). For comparison, the expression levels of miRNAs in HMEC-1 cells (left panel) and untreated HMEC-1 cells (right panel) were arbitrarily set at 1. Results are presented as means ±SEM of five independent experiments (*$p < 0.05$, **$p < 0.01$).

(E) Equal amounts of total RNA from MVs and whole cells were analyzed by northern blotting for miR-150.

(F) Pre-miR-150 levels in HMEC-1 cells treated with/without THP-1 or 293T MVs. Results are presented as means ±SEM of three independent experiments.

(G) Western blot analysis of c-Myb from HMEC-1 cells treated with THP-1 MVs for 0, 3, 6, and 12 hr, respectively (left panel) or from HMEC-1 cells that were treated with/without THP-1 or 293T MVs (right panel). Results are representative data from three independent experiments. Pictures of the western blot assay were analyzed using Bandscan software, and a statistical analysis is presented below (means ±SEM).

(H) Western blot analysis of c-Myb in HMEC-1 cells that were untreated (lane 1) or treated with miR-150-deficient THP-1 MVs (lane 3), normal THP-1 MVs (lane 4), pre-miR-150 (lane 5), or scrambled negative control RNAs (lane 2) (left panel) and in HMEC-1 cells that were treated with an unrelated pre-miRNA (lane 2), anti-miR-150 (lane 3), or scrambled negative control RNAs (lane 1) (right panel). Results are representative data from three independent experiments. Pictures of the western blot assay were analyzed using Bandscan software, and a statistical analysis is presented below (means ±SEM) (see also Figure S3).

of HMEC-1 cells (Figure 4C), while miR-150-sufficient 293T MVs enhanced HMEC-1 cell migration (Figure S4B). HMEC-1 cells were also directly treated with pre-miR-150, anti-miR-150, or siRNA against c-Myb, followed by assessment of HMEC-1 migration. Elevated exogenous miR-150 in HMEC-1 cells significantly enhanced cell migration (Figure 4C). Likewise, direct knockdown of c-Myb in HMEC-1 cells by siRNA (efficient interference of c-Myb expression was shown in Figures S4C and S4D) significantly increased cell migration (Figure 4C). Taken together, these results clearly demonstrate that secreted miRNAs present in MVs can be effectively delivered to cultured cells, where they function as endogenous miRNAs.

High Levels of Exogenous miR-150 from Plasma of Patients with Atherosclerosis Enhance Recipient HMEC-1 Cell Migration

The observation that miR-150 secreted by THP-1 cells can regulate HMEC-1 cell transmigration was further confirmed in an in vivo study. DiI-C16-labeled MVs isolated from THP-1 cells were injected intravenously into C57BL/6 mice, and the endothelium of mouse blood vessels was isolated and viewed by fluorescent microscopy. Figure 5A showed that the endothelium of mouse blood vessels was fluorescently labeled (bright red dots indicated by arrows). Moreover, THP-1 MVs were injected intravenously into C57BL/6 mice, and the expression levels of specific miRNAs, especially miR-150, in mouse blood vessels were assessed. At 6 hr postinjection, miR-150 was upregulated in mouse blood vessels, while injection of 293T MVs, which contain low levels of miR-150, had no effect on its expression (Figure 5B). These results suggest that secreted miRNAs present in MVs can be delivered into target cells and tissues under physiological conditions. Further, we found that MVs from atherosclerosis patient plasma had an increased level of miR-150 (Figure 5C) and that treatment of HMEC-1 cells with MVs from these patients with high levels of miR-150 decreased c-Myb protein level and enhanced cell migration (Figures 5D–5F). These results suggest that elevated secreted miR-150 may play a role in regulating endothelial cell function.

DISCUSSION

Since we have systematically discovered that miRNAs are stably present in animal serum/plasma and can serve as a class of biomarkers for cancers and other diseases (Chen et al., 2008), a large number of studies have also reported that circulating miRNAs can serve as biomarkers for various diseases (Resnick et al., 2009; Ng et al., 2009; Wang et al., 2009). However, the biological and physiological functions of circulating miRNAs remain completely unknown. Skog and coworkers (Skog et al., 2008) showed that exosomes from cultured cells contain miRNAs, suggesting that cell-derived MVs might serve as efficient carriers for delivery of circulating miRNAs. In the present study, we have characterized the possible carrier of circulating miRNAs. Our results suggest that MVs from whole human plasma are a mixture of microparticles (diameter > 100 nm) (Simons and Raposo, 2009), exosomes (diameter around 40–100 nm, density in the range of 1.13–1.19) (Simons and Raposo, 2009), and other vesicular structures and demonstrate that many types of MVs present in plasma contain miRNAs. Unlike MVs present in plasma, MVs released by cultured THP-1 cells are of uniform size, around 30–60 nm. It appears likely that MVs from cultured THP-1 cells represent primarily exosome-like vesicles, a finding that concurs with the findings of others (Cocucci et al., 2009; Simons and Raposo, 2009; Gibbings et al., 2009). We compared the ratio of miRNA expression in MVs to that in MV-free plasma and found that the majority of circulating miRNAs were present in MVs (Figure 1F). Interestingly, not all miRNAs were enriched in MVs; miR-223, miR-25, miR-451, and miR-181a showed relatively higher levels in MV-free plasma. These results show that circulating miRNAs are not exclusively stored in secreted MVs. It will be interesting to study the mechanisms that govern the release of miRNAs from circulating blood cells and cultured cells in further studies. However, in the present study, we focused on investigation of the potential function of secreted miRNAs in MVs.

Selective packaging of miRNAs into MVs is crucial to the specificity of biological function of secreted miRNAs. If miRNAs are released passively via broken cells, there would be no selection, and the ratio of released miRNAs would be similar to that in the cells. However, our results have demonstrated that cells selectively package miRNAs into MVs in circulating blood cells and in cultured THP-1 cells under various stimuli (Figure 2). In circulating blood cells, LPS altered the types and levels of miRNAs in MVs (Figure 2A), suggesting that blood cells selectively package miRNAs into MVs. However, it is also possible that the change of miRNA expression in MVs that we observed is due to a specific type of cell in whole blood that responds strongly to LPS stimulation and nonselectively packages its specific miRNAs into MVs. To rule out this possibility, we tested the profile change of miRNAs in MVs derived from individual cell lines under different stimuli. LPS, H_2O_2, AGEs, and OA/PA are classic acute and chronic inflammatory factors that alter macrophage function (Osterud and Bjorklid, 2003; Dale et al., 2008; Kashyap et al., 2009; Mandrekar et al., 2009; Takahashi et al., 2009). All four stimuli significantly altered miRNA types and levels in secreted THP-1 MVs, and miRNA alteration in MVs (Figures 2C–2E) is remarkably more significant than that in THP-1 cells (Figures 2F–2H). These results clearly demonstrate that cells can selectively package miRNA into MVs and actively secrete them into circulation for specific functions. Because miRNAs can be actively secreted by cells in response to various stimuli, our results also suggest that it would be necessary to assess the miRNAs both in cells and in secreted MVs when measuring alterations of miRNAs by stress factors. Furthermore, because the release of certain miRNAs by monocytes/macrophages is enhanced by various inflammatory stimuli, secreted miRNAs from monocytes/macrophages may represent a class of inflammatory factors involved in the inflammatory process.

miR-150 is abundantly expressed in monocytes (Xiao et al., 2007). It has been reported that this monocytic miRNA was altered under various conditions, including the immune response (Tsitsiou and Lindsay, 2009) and several diseases (Amaral et al., 2009; Bruchova et al., 2008; Garzon and Croce, 2008; Ohlsson Teague et al., 2009). miR-150 has also been reported to be involved in cell proliferation, migration, differentiation, and embryonic development (Xiao et al., 2007; Lin et al., 2008; Kopecki et al., 2007). One of the target genes of miR-150 is

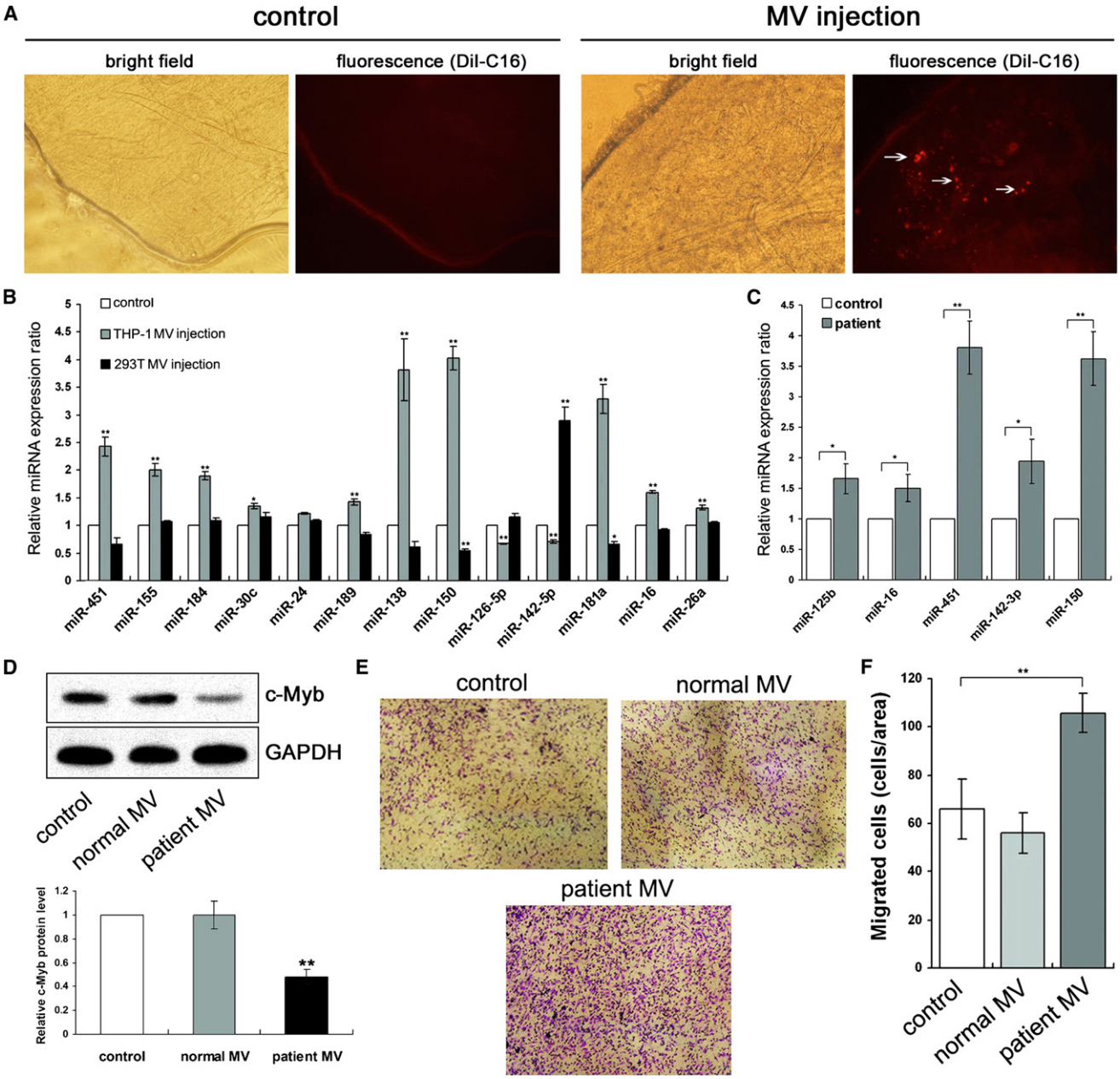

Figure 5. Pathophysiological Relevance of MV-Contained miR-150 in Circulation

(A) Image of the internalization of fluorescently labeled MVs into the endothelium of mouse blood vessels. DiI-C16-labeled MVs isolated from THP-1 cells were injected intravenously into C57BL/6 mice. After 6 hr, the endothelium of mouse blood vessels was isolated, washed, and then viewed under fluorescence microscopy.

(B) Comparison of miRNA expression in blood vessels isolated from mice after intravenous injection with THP-1 or 293T MVs or saline solution (control). For comparison, the expression levels of miRNAs in blood vessels of the saline group were arbitrarily set at 1. Results are presented as means ±SEM of six independent experiments (*$p < 0.05$, **$p < 0.01$).

(C) Comparison of miRNA expression in MVs from five pairs of patients with severe atherosclerosis and healthy donors. For comparison, the expression levels of miRNAs in MVs from healthy controls were arbitrarily set at 1. Results are presented as means ±SEM (*$p < 0.05$, **$p < 0.01$, patient versus control).

(D) Western blot analysis of c-Myb in HMEC-1 cells that were treated with/without normal or patient MVs. Results are representative data from three independent experiments. Pictures of the western blot assay were analyzed using Bandscan software, and a statistical analysis is presented below (means ±SEM).

(E) Photoimages of Transwell analysis of HMEC-1 cells that were treated with/without normal or patient MVs. Results are representative data from three independent experiments.

(F) Migration rates of HMEC-1 cells that were treated with/without normal or patient MVs. Results are presented as means ±SEM of three independent experiments (**$p < 0.01$, patient MVs versus control MVs).

c-Myb, a transcription factor related to cell proliferation, apoptosis, and tumorigenesis (Xiao et al., 2007). In this study, we demonstrated that exogenous miR-150 delivered by MVs from inflamed THP-1 cells or plasma of patients with severe atherosclerosis downregulates c-Myb in recipient HMEC-1 cells (Figures 3G, 3H, and 5D) and enhances HMEC-1 cell migration (Figures 4, 5E, and 5F). A recent study by Gibbings et al. (2009) showed that miRNAs could repress the mRNAs incorporated into the exosomes. By tracing the THP-1 cell-secreted miR-150 and characterizing its functional roles in HMEC-1 cells, our results demonstrated that miRNAs in MVs can also alter the gene in the target cells. This is direct evidence that a secreted miRNA specifically regulates a biological function of the recipient cells. Our results can therefore be taken to show that secreted miRNAs in circulation have a great potential to become a class of signaling molecules that mediate intercellular/interorgan communication. Furthermore, given that miR-150 in MVs derived from the plasma of patients with severe atherosclerosis downregulates c-Myb and enhances cell migration in recipient HMEC-1 cells and that the migration of endothelial cells lining blood vessels is a major component of atherosclerosis, it appears likely that elevated miR-150 levels in MVs could mediate crosstalk between circulating monocytes/macrophages and vascular endothelial cells under various pathophysiological conditions. Such crosstalk mediated by miRNAs in MVs provides a mechanism that may underlie vascular injury under various dysfunctional conditions, including obesity, hyperglycemia, and chronic inflammation.

Another interesting finding in the present study is that we have observed an effective delivery of human miRNAs into murine cells through cell-derived MVs. The results suggest a cross-species delivery of miRNAs via MVs. However, the mechanism underlying this cross-species delivery of miRNAs is completely unknown. It is not clear whether the cross-species delivery of miRNAs is specific and mediated by protein-protein interactions or nonspecific and mediated by lipid-carbohydrate interactions. Our observation of effective delivery of human miRNAs into murine cells via MVs derived from various human cells may imply that exosomes possess highly conserved ligands for conserved receptors. However, although previous studies (Théry et al., 2006; Johnstone, 2006) show that exosomes derived from different cell types do share many common properties, no such conserved molecules that mediate the interactions between exosomes and recipient cells have been identified yet.

Since their discovery in *C. elegans* 15 years ago, miRNAs have been implicated in a wide array of cellular and developmental processes (Ambros, 2004; Bartel, 2004). To date, more than 500 miRNAs are predicted to be expressed in humans, and nearly 30% of genes are predicted to be regulated by miRNAs (Ambros, 2004; Bartel, 2004). The present study extends our understanding of the role of miRNAs by illustrating that miRNAs can be secreted and delivered into target cells and that these exogenous miRNAs can alter the cellular functions of the recipient cells by modulating the expression of their target genes. From this point of view, secreted miRNAs may represent a class of signaling molecules that play an important role in mediating intercellular communication. Moreover, the secretion and targeting of miRNAs among the different cells establishes a highly regulated complex network under various physiological and pathophysiological conditions. Compared to classic cellular communications mediated by hormone/cytokine-receptor and antigen-antibody interactions that generally occur only in certain types of cells, involve a single or few molecules, and affect target cells in a "one-way" fashion, secreted miRNA-based cell communication has the potential to function for every type of cell, to deliver many types of miRNAs with each miRNA targeting multiple genes, and to affect target cells in "two-way" or "multi-way" fashion. Investigating this secreted miRNA-mediated shift of cell communication from a "cable telephone" to "cell phone" fashion would help us to further understand the nature of biological signaling, discover novel mechanisms of disease, and develop new therapeutic strategies.

EXPERIMENTAL PROCEDURES

Reagents, Cells, and Antibodies

The human monocyte/macrophage cell line THP-1 was purchased from China Cell Culture Center (Shanghai, China); HMEC-1 cells were provided by E.W. Ades (Centers for Disease Control and Prevention; Atlanta). HMEC-1 cells were grown in MCDB-131 (Invitrogen; Carlsbad, CA) supplemented with 10 ng/ml epidermal growth factor (Becton Dickinson; San Jose, CA), 10 ng/ml hydrocortisone (Sigma Chemical, St. Louis), and 10% fetal bovine serum (FBS) (GIBCO; Carlsbad, CA). HMEC-1 cells were seeded on gelatin (Difco, Detroit)-coated tissue culture plates or permeable Transwell filters (8.0 μm pore size, Costar; Cambridge, MA). THP-1 cells were cultured in standard RPMI 1640 medium supplemented with 10% FBS (GIBCO) in a 5% CO_2, water-saturated atmosphere. Anti-C-Myb (C19) and anti-GAPDH (6C5) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Synthetic RNA molecules, including pre-miR-150, anti-miR-150, and scrambled negative control oligonucleotides (pre-ncRNA and anti-ncRNA), were purchased from Ambion (Austin, TX).

Blood Collection

Method for collecting blood is described in the Supplemental Experimental Procedures.

MV Isolation

MVs were isolated from the plasma of healthy donors or of patients with atherosclerosis and from cell culture medium by differential centrifugation according to previous publications (Skog et al., 2008; Valadi et al., 2007). As shown in the schematic description of the experimental procedure (Figure S5), after removing cells and other debris by centrifugation at 300 × *g*, 1200 × *g*, and 10,000 × *g*, the supernatant was centrifuged at 110,000 × *g* for 2 hr (all steps were performed at 4°C). MVs were collected from the pellet and resuspended in FBS-free medium.

OptiPrep-based density gradient centrifugation was conducted as previously described (Bartz et al., 2008) (see Supplemental Experimental Procedures).

Cryo-Electron Microscopy and Transmission Electron Microscopy

Detailed methods for performing cryo-EM and TEM are described in the Supplemental Experimental Procedures.

RNA Isolation and Quantitative RT-PCR of Mature miRNAs

Total RNA of MVs derived from 10^8 cells was extracted using TRIzol Reagent (Invitrogen). Quantitative RT-PCR was carried out using TaqMan miRNA probes (Applied Biosystems; Foster City, CA) according to the manufacturer's instructions. Briefly, 5 μl of total RNA was reverse transcribed to cDNA using AMV reverse transcriptase (TaKaRa; Dalian, China) and a stem-loop RT primer (Applied Biosystems). Real-time PCR was performed using a TaqMan PCR kit on an Applied Biosystems 7300 Sequence Detection System. All reactions, including no-template controls, were run in triplicate. After the reaction, the

C_T values were determined using fixed threshold settings. To calculate the absolute expression levels of target miRNAs, a series of synthetic miRNA oligonucleotides at known concentrations were also reverse transcribed and amplified. The absolute amount of each miRNA was then calculated by referring to the standard curve. In the experiments presented here, miRNA expression in cells is normalized to U6 snRNA, as is done in many other reports. However, because there is no current consensus on the use of housekeeping genes for quantitative RT-PCR analysis in MVs and the expression level of U6 snRNA is very low in MVs (data not shown), the expression levels of target miRNAs in MVs were directly normalized to the total protein content of MVs in our study.

Fluorescence Labeling of MVs and Confocal Microscopy

THP-1 cells were labeled with DiI-C16 for 1 hr and then washed three times with PBS. The cells were resuspended and cultured overnight in RPMI 1640 medium supplemented with 10% FBS. The supernatants were then collected and centrifuged to harvest MVs. THP-1 MVs were resuspended in MCDB-131 medium and incubated with cultured HMEC-1 cells. After incubation for various length of time, HMEC-1 cells were washed, fixed, and observed under confocal microscopy (FV1000; Olympus, Tokyo). The pictures were taken under these conditions: Objective Lens: PLAPON 60X O NA: 1.42; Scan Mode: XY; Excitation Wavelength: 405 nm for DAPI and 543 nm for DiI-C16; Image Size: 1024 × 1024 Pixel.

Cell Transfection with ncRNA, Anti-miR-150, or Pre-miR-150

THP-1 or HMEC-1 cells were seeded on 60 mm dishes and were transfected the following day using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For overexpression of miR-150, 400 pmol of pre-miR-150 or scrambled negative control pre-miRNA (pre-ncRNA) was used. For knockdown of miR-150, 200 pmol of anti-miR-150 or scrambled negative control anti-miRNA (anti-ncRNA) was used. Cells were harvested 24 hr after transfection.

Endothelial Cell Migration Assay

The migration ability of HMEC-1 was tested in a Transwell Boyden Chamber (6.5 mm, Costar). The polycarbonate membranes (8 μm pore size) on the bottom of the upper compartment of the Transwells were coated with 0.1% gelatin matrix. Cells were suspended in serum-free MCDB-131 culture medium at a concentration of 4×10^5 cells/ml, treated with or without THP-1 MVs for 2 hr and then added to the upper chamber (4×10^4 cells/well). Simultaneously, 0.5 ml of MCDB-131 with 10% FBS was added to the lower compartment, and the Transwell-containing plates were incubated for 4 hr in a 5% CO_2 atmosphere saturated with H_2O. At the end of the incubation, cells that had entered the lower surface of the filter membrane were fixed with 90% ethanol for 15 min at room temperature, washed three times with distilled water, and stained with 0.1% crystal violet in 0.1 M borate and 2% ethanol for 15 min at room temperature. Cells remaining on the upper surface of the filter membrane (nonmigrant) were scraped off gently with a cotton swab. Images of migrant cells were captured by a photomicroscope (BX51, Olympus). Cell migration was quantified by blind counting of the migrated cells on the lower surface of the membrane, with five fields per chamber.

Northern Blotting Analysis

Northern blot method is described in the Supplemental Experimental Procedures.

Plasmid Construction and Luciferase Assay

Detailed methods are described in the Supplemental Experimental Procedures.

Western Blotting

c-Myb protein levels were quantified by western blot analysis of whole-cell extracts using antibodies against c-Myb. Normalization was performed by blotting the same samples with an antibody against GAPDH.

Animals

All animal models were maintained in a C57BL/6 background on a 12 hr light/dark cycle in a pathogen-free animal facility at Nanjing University. The Institutional Review Board of Nanjing University approved all housing and surgical procedures. At 8 weeks of age, mice received tail-vein injections of saline or normal THP-1 MVs or DiI-C_{16}-labeled MVs. After 6 hr, murine thoracic aorta endothelium was isolated, washed with PBS five times to remove contaminated exosomes, and then viewed under fluorescence microscopy. For measurement of miRNA levels, total RNA was extracted from thoracic aorta by using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions.

Statistical Analysis

All photoimages of western blotting and semiquantitative RT-PCR are representative of at least three independent experiments. Quantitative RT-PCR and cell migration assays were performed in triplicate, and each experiment was repeated several times. Data shown are presented as means ±SEM of at least three independent experiments; differences are considered statistically significant at $p < 0.05$ using Student's t test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two tables, and five figures and can be found with this article online at doi:10.1016/j.molcel.2010.06.010.

ACKNOWLEDGMENTS

We would like to thank the Center for Biological Imaging, Research Platform for Protein Sciences Research, Chinese Academy of Sciences for our electron microscopy work, and we are grateful to Gang Ji and Shufeng Sun for their help with making EM samples and taking EM images. We also thank Zhenyou Zou for his technical assistance. This work was supported by grants from the National Natural Science Foundation of China (90813035, 30890044, and 30570731), National Basic Research Program of China (973 Program) (2006CB503908, 2006CB503909, 2004CB518603, and 2003CB715905), the "111" Project, and the Natural Science Foundation of Jiangsu Province (BK2004082 and BK2006714).

Received: October 13, 2009
Revised: January 27, 2010
Accepted: April 29, 2010
Published: July 8, 2010

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